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14. ABSTRACT The goal of this Exploration-Hypothesis Development Award is to analyze the role of NF1 in the functional expression of CD1d. During the second year of the award, we have analyzed the ability of bone marrow-derived dendritic cells (BMDC) from wildtype (WT) and NF1+/- mice to stimulate fresh NKT cells. The original hypothesis was that we would observe more Th2 (i.e., anti-inflammatory)--as opposed to Th1 (i.e., pro-inflammatory)--cytokine production by NKT cells. Our results suggest that NKT cells from both WT and NF1+/- mice can produce both Th1 and Th2 cytokines at comparable levels. As indicated last year, when analyzing the level of cell surface CD1d, it was observed that BMDC generated from NF1+/- mice expressed ~75% of the WT level of CD1d, but were able to stimulate NKT cells as well as those from WT mice. However, we have found a reduction in a B220+CD1d-hi population in splenocytes from NF1 +/- mice. This suggests that NF1 plays an important role in the qualitative expression of CD1d. Furthermore, NKT cells from NF1+/- mice are more activated at baseline, than those from WT mice. In line with this observation, RMA/S tumor growth in NF1+/-mice was reduced compared to WT mice. Therefore, these data strongly suggest that NF1 plays an important (albeit inhibitory) role in the host's innate antitumor immune response (i.e., CD1d/NKT cell axis).					
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INTRODUCTION: Neurofibromatosis type 1 is a disease that affects one in 2500 – 3500 individuals caused by a deficiency in the gene encoding the Ras-GAP, NF1 (1-3). As a result, a number tumors including those of the central nervous system (schwannomas and gliomas), and the development of other malignancies, including myeloid leukemias (e.g., juvenile myelomonocytic leukemia in children) frequently develop, as can T cell leukemias and lymphomas (4-6). The *NF1* gene encodes the Ras GTPase activating protein, neurofibromin, which normally inhibits signaling via the Ras-Raf-MEK-ERK pathway (1, 2). In the absence of (or reduction in) NF1, hyperactivation of Ras-Raf-MEK-ERK pathway signaling and constitutive growth activation (and hyperproliferation) of such NF1-deficient cells occurs (7, 8). Signal transduction pathways are not only important for cell growth, but also regulate immune responses. We have found that the ERK1/2 MAPK pathway is important in the control of antigen presentation by a molecule called CD1d (9). CD1d molecules present lipids to a unique subpopulation of NKT cells called "NKT cells" (10), resulting in both pro-inflammatory and anti-inflammatory cytokine production. Inhibition of the ERK1/2 pathway causes a substantial impairment of CD1d-mediated antigen presentation and altered intracellular trafficking of CD1d (9). Thus, ERK1/2 is important for the functional expression of CD1d. Our preliminary data showed that bone marrow-derived dendritic cells (ideal antigen presenting cells for NKT cells) from NF1^{+/-} mice were better stimulators of NKT cells than those from WT mice (Fig. 1). Further, consistent with that results, we found that NF1^{+/-} mice have more NKT cells from WT mice (Fig. 2). Thus, we proposed the following hypothesis to be tested in this project: **NF1 controls the functional expression of CD1d by regulating ERK1/2 signaling, resulting in enhanced anti-inflammatory cytokine production by NKT cells.** Such a result would be consistent with *in vivo* observations in both T and B lymphoma (11, 12) and tumor recurrence (13-18) models.

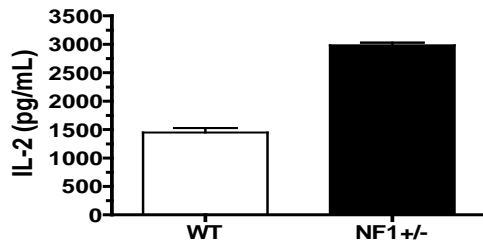


Fig. 1. Enhanced NKT cell stimulation by NF1^{+/-} BMDC. BMDC were generated from wildtype and NF1^{+/-} C57BL/6 mice and then cocultured with the NKT cell hybridoma, N37-1A12, for 24 hr. Supernatants were harvested and IL-2 production was measured by ELISA.

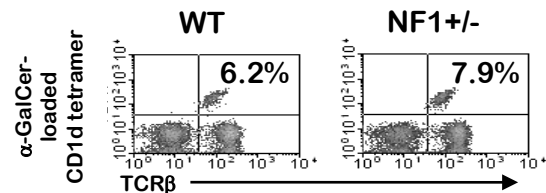


Fig. 2. Liver NKT cells in WT and NF1^{+/-} mice. Hepatic MNC were harvested from WT and NF1^{+/-} C57BL/6 mice and stained with a TCRβ-specific mAb and α-GalCer-loaded mouse CD1d tetramer to identify NKT cells. Analysis was by flow cytometry.

BODY: Our studies to analyze the mechanism(s) by which NF1 regulates antigen presentation by CD1d have progressed very well since the award of this grant in January 2010, with significant progress being made. However, an important initial obstacle we faced was the inability of Dr. Masood Khan, the original postdoctoral fellow assigned to this work, to return to the United States from his visit home to India. Instead, I reassigned this project to a very talented postdoctoral fellow in my laboratory, Dr. Jianyun Liu. Dr. Liu is a highly productive postdoctoral fellow who has been training under my direction since 2006. Once it was apparent that Dr. Khan would be unable to return to the United States, Dr. Liu was able to essentially hit the ground running, learning the additional techniques required for the successful completion of this project in a relatively short period of time. She has continued to do very well; we have been highly successful, and I have been extremely pleased with our results and their potential for future applications. Our progress to date is indicated below:

Specific Aim #1: Analyze the mechanism(s) by which NF1 regulates antigen presentation by CD1d.

Task 1. Analyze the functional expression of CD1d on WT and NF1 +/- BMDC (months 1 – 12):

- 1a.** Generate BMDC from WT and NF1 +/- mice (5 mice/group x 2 experiments) and test their ability to stimulate cytokine production in a CD1d-dependent manner from NKT cell lines derived from WT mice *in vitro*
- 1b.** Assess the cytokines (e.g., IL-4, IL-13, IFN- γ) produced by fresh hepatic and thymic NKT cells from WT and NF1 +/- mice (5 mice/group x 2 experiments) in BMDC/NKT cell co-cultures (months 4 – 6)

In our preliminary data provided for the original submission of this grant, we demonstrated that Type II NKT cell hybridomas could be activated by BMDC from NF1 +/- mice at a higher level than those from WT mice. This was not the case when we used canonical (i.e., Type I) NKT cell hybridomas. From those results, our hypothesis was that NF1 +/- BMDC would be able to stimulate cytokine release that was more biased towards a Th2 phenotype (i.e., IL-4 or IL-13), rather than a Th1 profile (e.g., IFN- γ). As we reported last year, we analyzed the ability of BMDC from both WT and NF1 +/- mice to stimulate NKT cells from WT mice, and observed lower levels of Th2 cytokines by WT NKT cells, when cocultured with NF-1 +/- BMDC (with a greater reduction in IL-4 than IL-13; Fig. 3). More recently, we have looked at IFN- γ production by NKT cells from a larger number of WT and NF1 +/- mice. Consistent with the data in Fig. 3 below, our newer experiments

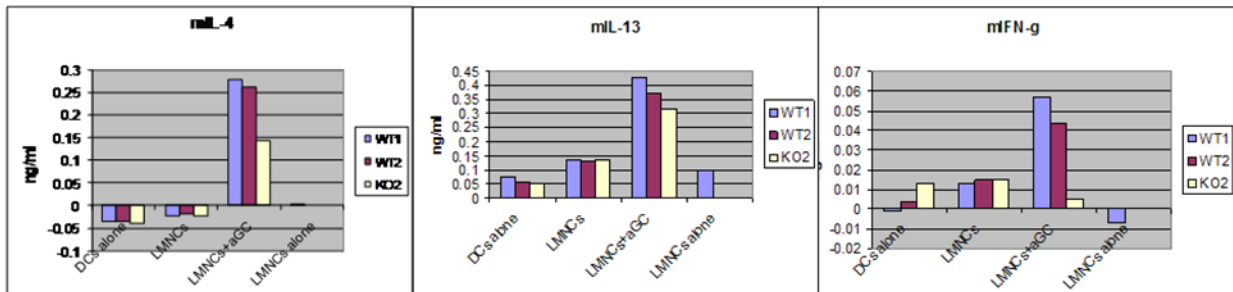


Fig. 3. BMDCs (5×10^5 /well) from WT or NF1 +/- (KO2) mice were fixed in 0.05% paraformaldehyde and co-cultured with liver mononuclear cells from WT mice as a source of NKT cells (LMNCs, E:T=1:2, 48 hours of culture). When co-cultured with LMNCs, 200 ng/ml of alpha-GalCer (α GC) was added to some of the cultures. The supernatants were harvested for measurement of the indicated cytokines by ELISA.

clearly demonstrated that there were consistently higher levels of IFN- γ produced by NKT cells from NF1 +/- mice (Fig. 5). This result is very exciting and, as described later in this report, this higher IFN- γ production appears to translate into more effective antitumor immunity in these animals. These data will be presented in the next report.

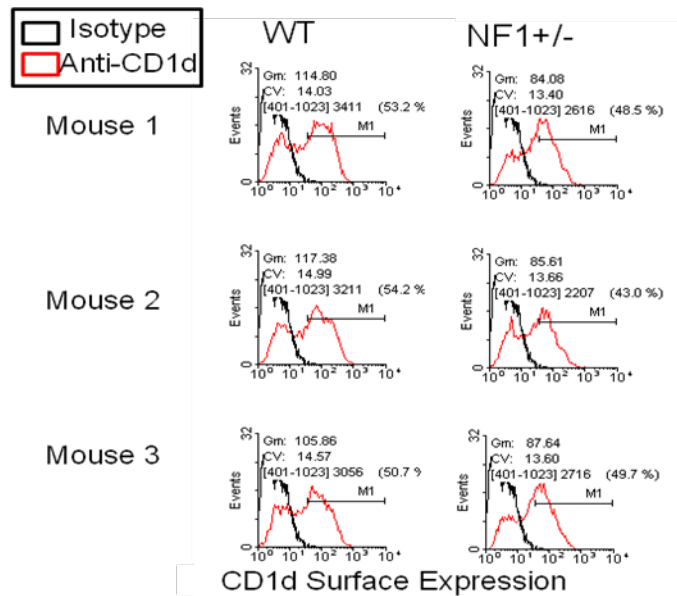


Fig. 4. Less CD1d surface expression on NF1+/- compared to WT BMDC. WT and NF1+/- BMDC were stained with a PE-labeled isotype control or PE-conjugate anti-mouse CD1d antibody (1B1). Analysis was by flow cytometry.

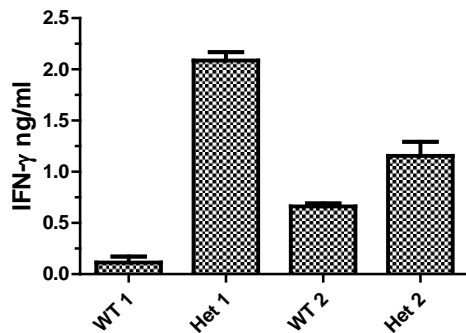


Fig. 5. NKT cells from NF1 +/- mice secrete more IFN- γ than those from WT mice. Liver mononuclear cells from WT and NF1+/- mice were isolated and cocultured with α -GalCer-pulsed MC57G cells expressing the mouse CD1d cDNA. After a 48 hr incubation period, the supernatants were harvested and IFN- γ production was measured by ELISA.

Of additional important note, we found over multiple experiments that the level of CD1d on NF1+/- BMDC is actually lower than that on WT BMDC (Fig. 4). Thus, CD1d on NF1+/- cells is approximately 75% of that found on WT BMDC. However, there was not a substantial reduction in NKT cell IL-13 production when cocultured with NF1+/- BMDC compared to WT (Fig. 3). Therefore, considering the dramatic decrease in IFN- γ secretion observed, this suggests that the reduced CD1d expression on NF1+/- BMDC is likely not the main reason behind the results seen. Instead, we would argue that there is a "qualitative" difference in the functional expression of CD1d between NF1+/- and WT BMDC, resulting in the data observed.

Task 1c. Perform confocal microscopy on BMDC from WT and NF1 +/- mice (5 mice/group x 2 experiments) for the analysis of CD1d intracellular trafficking (months 7 – 8)

Task 1d. Analyze CD1d internalization from the surface and recycling to and from the cell surface (months 9 – 10)

Task 1e. Perform Western blot analysis of MAPK (especially ERK1/2) signaling pathways in BMDC from WT and NF1+/- mice (5 mice/group x 2 experiments; months 11 – 12). These experiments are in progress and will be completed very soon.

This past year, and in line with experiments for **Task 1e**, we have performed analyses of the activation of MAPK in tissues from WT and NF1+/- mice. The expectation is that the differences in the ability of BMDC to stimulate NKT cells reported last year, would be reflected in altered MAPK phosphorylation. Thymocytes and splenocytes from WT and NF1+/- mice were isolated, lysed and the activation of ERK1/2 and JNK was determined by Western blot analysis. Notably, there was a higher level of both ERK1/2 (Fig. 7) and JNK (Fig. 8) phosphorylation in tissues from NF1+/-, as compared to WT mice.

We analyzed the recycling rates in BMDC from WT and NF1+/- mice. There was no difference between the two sets of mice (Fig. 6), suggesting that the differences between these mice are not at the level of recycling.

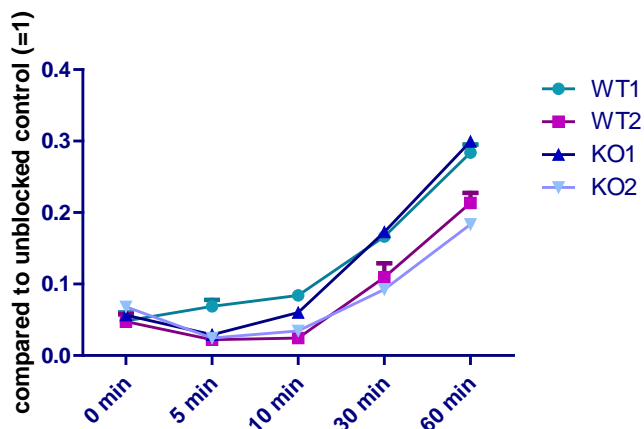


Fig. 6. Similar recycling rate of CD1d molecules in NF1+/- BMDCs. BMDCs from NF1+/- mice or their WT littermates were pretreated with the protein synthesis inhibitor cyclohexamide. The cells were then blocked with CD1d antibody (1B1) on ice, and then incubated at 37°C for the indicated time intervals. Newly recycled surface CD1d molecules were labeled by PE-conjugated 1B1 and analyzed by flow cytometry. The ratios of MFI of CD1d at each time points to unblocked cell control (=1) are calculated. Representative data from three independent experiments are shown.

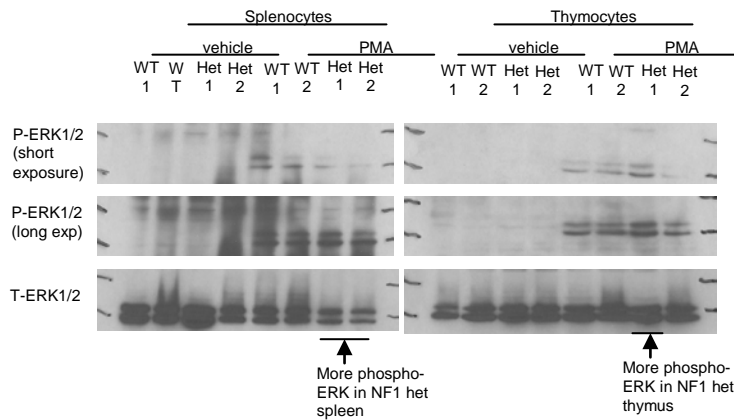


Fig. 7. Greater activation of ERK1/2 in NF1^{+/+} as compared to WT mice. splenocytes and thymocytes from WT and NF1^{+/+} mice were treated for 30 min with vehicle or PMA (100 ng/ml), lysed and analyzed for the activation of ERK1/2 by Western blot analysis. The blots were stripped and re probed for total ERK1/2.

During the course of the experiments reported last year, we observed a series of surprising results we believe are quite exciting: Female mice have more NKT cells (on a percentage basis) than male mice; this is the same whether we are using WT or NF1^{+/+} mice. Additionally, BMDC derived from female NF1^{+/+} mice appeared to be better antigen presenting cells to NKT cells than are those from male NF1^{+/+} mice. This has not been apparent with WT BMDC. It is important to note here that all of our experiments compared WT and NF1^{+/+} mice that were age- and sex-matched. With the unexpected observation of a sex difference in NKT cells (and NF1^{+/+} BMDC), we decided to breed additional NF1^{+/+} mice so that we can include this direct comparison for **Tasks 1c, 1d and 1e**. These experiments are ongoing and will allow us to be able to compare BMDC from male and female NF1^{+/+} mice. This will not only tell us if there are differences in the intracellular trafficking and recycling capacity of CD1d between WT and NF1^{+/+} BMDC, but also if MAPK signaling varies between these two sets of BMDC. These experiments will also demonstrate if there are differences in these activities between BMDC derived from males and females. The studies to address possible sex differences will be incorporated into the same experiments to both maintain the originally projected number of mice, as well as accomplish those subtasks in a shorter period of time. Looking at the larger picture, these studies may reveal fundamental sex differences in the cell biology and biochemistry of the innate antitumor immune response, in addition to the role for NF1 in such activities. Such studies would be important in subsequent grant applications.

Milestone #1: Approval by IACUC of adding NF1^{+/+} mice to the existing animal RMA/S tumor protocol for the *in vivo* experiments proposed in Specific Aim #2. This was accomplished and animal studies are ongoing.

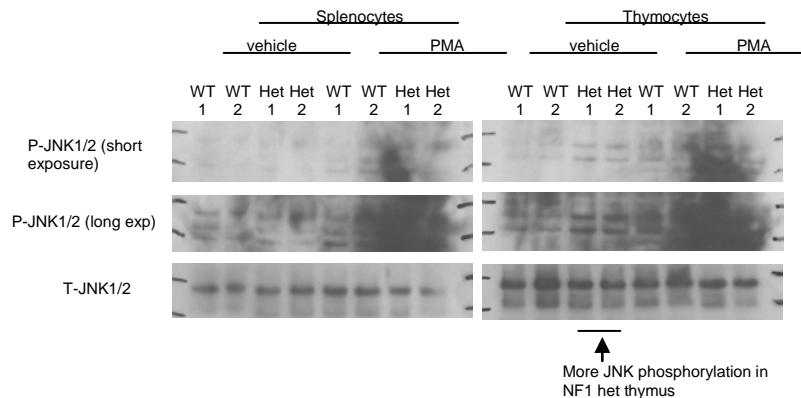


Fig. 8. Greater activation of JNK1/2 in NF1^{+/-} as compared to WT mice. splenocytes and thymocytes from WT and NF1^{+/-} mice were treated for 30 min with vehicle or PMA (100 ng/ml), lysed and analyzed for the activation of JNK1/2 by Western blot analysis. The blots were stripped and reprobed for total JNK1/2.

Due to our desire to include the male vs. female NF1^{+/-} BMDC experiments to accomplish subtasks 1c, 1d and 1e, which requires breeding of more mice, we accomplished Milestone #1 early last year and we completed the experiments for Task 2a. This will allow us to continue on track to accomplish both aims and Milestones along the proposed timeline indicated in the original application.

Specific Aim #2: *Analyze the in vivo growth of hematopoietic tumors in wildtype and NF1 +/- mice.*

Task 2. Analyze the growth of RMA/S tumors *in vivo*:

2a. Inoculate various numbers of RMA/S-V and RMA/S-CD1 tumor cells (1, 5 and 10 x 10⁵ cells, i.p.) into WT and NF1^{+/-} mice to test the differences in susceptibility to tumor growth (months 13 – 16)—25 mice in total. This task is completed.

Milestone #2: Optimum tumor inoculum size for subsequent experiments established.

Tasks 2b, c and d are ongoing and will be completed soon. Preliminary data suggest that NF1^{+/-} mice have lower tumor burdens than WT mice in RMA/S tumors that are CD1d⁺ (Fig. 9).

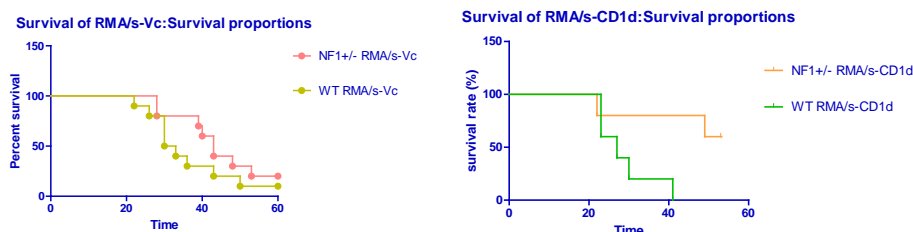


Fig. 9. Increased antitumor activity against CD1d⁺ tumors in NF1^{+/-} mice. NF1^{+/-} mice and their WT littermates were inoculated with 5x10⁵ RMA/S-V or RMA/S-CD1 cells, i.p. The mice were monitored for up to 60 days post-tumor inoculation. Representative data of two independent experiments are shown. P<0.05 in right graph.

Year 2 Annual Report Exploration-Hypothesis Development Award
“Regulation of CD1d-Mediated Antigen Presentation by NF1”
Contract #: W81XWH-10-1-0048/Grant # NF093069
Randy R. Brutkiewicz (Principal Investigator)

Milestone #3: Submit manuscript for publication. We have already begun to write a manuscript based on the CD1d levels and cytokine release by NKT cells from WT and NF1+/- mice. The other analyses should comprise another manuscript. We therefore anticipate at least two manuscripts from the work performed for this project.

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KEY RESEARCH ACCOMPLISHMENTS:

- Increased JNK and ERK activation in NF1+/- mice
- No significant difference in the number of iNKT cells between WT and NF1+/- mice.
- BMDCs from NF1+/- mice have lower surface expression of CD1d compared to those from WT mice.
- BMDCs from NF1+/- mice are able to stimulate NKT cells to the same level as BMDCs from WT littermates.
- NF1 +/- mice have a smaller splenic B220⁺CD1d^{hi} population.
- Liver mononuclear cells (LMNCs) from NF1+/- mice are more activated by CD1d compared to those from WT littermates.
- α -GalCer stimulated comparable levels of circulating cytokines in NF1 +/- and WT mice *in vivo*.
- Increased antitumor activity in NF1 +/- mice.
- A haploinsufficiency of NF1 causes a reduction in CD1d expression and modifies NKT cell function.

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REPORTABLE OUTCOMES:

None yet; however, we do expect to submit at least two manuscripts for publication based on this work in the next year.

CONCLUSION:

In our data generated to date, we have shown that BMDC from WT and NF1+/- mice differ in their ability to present antigen by CD1d to NKT cell hybridomas or fresh WT NKT cells. When we analyzed the cytokine profile of freshly-isolated WT NKT cells stimulated by WT or NF1+/- BMDC, we observed a substantial difference. Although there was a modest decrease in NKT cell IL-4 production induced by NF1+/- BMDC compared to WT BMDC, there was only a minor difference in IL-13 production. In stark contrast, NF1+/- BMDC were poor at inducing IFN- γ secretion from NKT cells. This suggests an important qualitative role for NF1 in regulating the functional expression of CD1d. Of additional significance, we did observe a modest (~25%) decrease in cell surface CD1d on NF1+/- BMDC relative to those from WT mice; however, this was not to the extent that could explain our NKT cell cytokine production data. These latter results suggest that NF1 also plays a role in the normal cell surface expression of CD1d.

Related to the cytokine data, we found that there is less tumor growth in NF1+/- as compared to WT mice. This is consistent with the higher IFN- γ production by the former group of mice. Furthermore, there is more ERK1/2 and JNK1/2 activation in splenocytes and thymocytes from NF1+/- mice.

Another exciting observation was that female mice have more NKT cells than male mice on a percentage basis. As this was the case in both WT and NF1+/- mice, this finding has more broad-ranging relevance beyond just NF1 and immune evasion. Such a discovery could have an important impact on potential treatments for NF1 and other conditions in which there are sex differences between NKT cells and/or the functional expression of CD1d.

"So what"?

Why are our results important? Alterations in an important arm of the innate immune response (i.e., IFN- γ production by NKT cells) could have a major impact in the host's antitumor immune response. Without a Th1 (i.e., pro-inflammatory) cytokine bias, a host cannot effectively mount a response against a tumor; in the case of this project, those tumors that have been found in patients with neurofibromatosis type 1. It is possible that IFN- γ is playing a protective role here. A glycolipid that has helped many of us in the CD1d field understand the importance of the CD1d/NKT cell axis, is α -galactosylceramide (α -GalCer). Most certainly, a future approach would be to include α -GalCer analogs that induce more of a pro-inflammatory cytokine profile than the parental compound or other α -GalCer analogs that induce an anti-inflammatory cytokine profile. These would enhance our IFN- γ studies reported here. Such analogs already exist (10) and could form the basis of the next steps beyond this current grant. Studies such as these could have wide-ranging applications for patients with neurofibromatosis type 1.

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Randy R. Brutkiewicz (Principal Investigator)

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